Journal of Medical Virology 54:1–6 (1998)

A Study of the Antigenicity and Immunogenicity of a New Hepatitis B Vaccine Using a Panel of Monoclonal Antibodies

J.A. Waters,[1]* C. Bailey,[2] C. Love,[3] and H.C. Thomas[1]

[1]*Department of Medicine, Imperial College School of Medicine at St. Mary's, St. Mary's Hospital, London, United Kingdom*
[2]*Medeva Group Development, Leatherhead, Surrey, United Kingdom*
[3]*Medeva Group Development, Evans Medical Ltd., Speke, Liverpool, United Kingdom*

The successful prevention of infection with hepatitis B virus (HBV) has been achieved by vaccination with purified hepatitis B surface antigen (HBsAg). The ability of a novel synthetic HBV envelope antigen vaccine (Hep B-3, Hepagene™; Medeva), which contains part of the pre-S1 and the complete pre-S2 regions and the whole of the S region and was produced in a mammalian cell line, to induce antibodies required for a protective immune response is of importance. In this study, the use of a panel of monoclonal antibodies known to bind to epitopes within the common ''a'' determinant has demonstrated that the epitopes present on this new vaccine are comparable to those found with plasma-derived HBsAg. In addition, the epitope specificity of the antibodies induced by this vaccine was examined and shown to accord well with previous results obtained using both a plasma-derived vaccine and a recombinant vaccine prepared in yeast. ***J. Med. Virol. 54:1–6, 1998.*** © 1998 Wiley-Liss, Inc.

KEY WORDS: hepatitis B surface antigen; common ''a'' determinant; antigenic structure

INTRODUCTION

The prevention of infection with hepatitis B virus (HBV) has been achieved successfully by vaccination with hepatitis B surface antigen (HBsAg). The original HBsAg vaccines were prepared from the plasma of HBV-infected asymptomatic carriers [Szmuness et al., 1980], but these have been superceded by recombinant vaccines produced in yeast [Valenzuela et al., 1982; McAleer et al., 1984]. Using monoclonal antibodies directed against the common "a" determinants of HBsAg, it was shown previously that the specificities of the antibodies induced by the recombinant vaccines were similar to those produced by the plasma-derived vaccine [Waters et al., 1987]. In other studies, the HBsAg epitopes detectable on the yeast-derived product were similar to those on HBsAg found in the plasma of infected individuals [Gerety, 1988]. The vaccine under study, in addition to the small HBsAg, contains pre-S2-S (the middle protein) and part of the pre-S1 inserted into the N-terminal region of the small HBsAg.

The ability of a new vaccine preparation, with an altered composition to induce antibodies of the specificity required for a protective response, is of importance. Immunisation with the current vaccines has shown that the immune response against the common "a" determinant protects against infection with HBV of either the *ay* or *ad* subtype [Le Bouvier et al., 1976; Koziol et al., 1976; Szmuness et al., 1980; Jilg et al., 1984]. Antibody recognition of the "a" determinant is partially sensitive to reduction of the disulphide bonds [Imai et al., 1974]. Tertiary structure, not dependent on disulphide bonds, is recognised also by polyclonal anti-HBs as solubilisation of HBsAg with nonionic detergent reduces antibody recognition [Howard et al., 1984]. However, treatment of HBsAg with sodium dodecyl sulphate (SDS), which releases a dimer of the glycosylated and nonglycosylated 25,000 dalton polypeptides, retains important "a" determinants [Mishiro et al., 1980; Waters et al., 1987].

In this study, a panel of monoclonal antibodies raised against HBsAg purified from a plasma pool which recognize four discrete epitopes and four overlapping epitopes of the common "a" determinant [Waters et al., 1991], has been used to investigate the epitopes present on a novel synthetic HBV envelope antigen formulated as the vaccine Hep B-3. This panel includes an

*Correspondence to: Department of Medicine, Imperial College School of Medicine at St. Mary's, St. Mary's Hospital, London W2 1NY, United Kingdom.

Accepted 3 August 1997

© 1998 WILEY-LISS, INC.

antibody, RFHBs 1, which was shown to protect against infection with HBV in the chimpanzee model [Iwarson et al., 1985].

The binding of this panel of monoclonal antibodies to two cyclical peptide analogues of the region of the surface antigen most likely to contain the common "a" determinant, p124–137 and p139–147, has been described previously [Waters et al., 1991]. RFHBs 1, which binds to p124–137; RFHBs 7, which binds to p139–147; and another antibody, RFHBs 18, which binds to neither peptide, were used to examine the specificity of the antibodies evoked by vaccination of human subjects with Hep B-3.

MATERIALS AND METHODS

Antigen and Vaccine

The recombinant hepatitis B vaccine, Hep B-3 (Hepagene™; Medeva), contains particles consisting of three protein monomers: S (major HBV envelope protein), pre-S2-S (middle protein), and pre-S1-S. This latter protein comprises an important antigenic component of pre-S1 (aa 20–47) inserted into the N-terminal region of the S protein. These proteins are expressed in continuous mouse C127I cells (ATCC CRL 1616, Rockville, MD), which have been used as host cells for expression of a number of recombinant gene products, including HBsAg [Yoneyama et al., 1988; Samonta and Youn, 1989].

The C127I cell line producing Hep B-3 was constructed by transfection with expression plasmids for the S, pre-S2-S, and pre-S1-S genes. In each expression plasmid, the genes were placed under the control of the mouse metallothionein promoter [Pavlakis and Hamer, 1983]. Cells were grown on microcarrier beads and the Hep B-3 particles secreted into the culture medium. Hep B-3 was purified from harvest medium ultrafiltration. Hep B-3 vaccine was formulated by adsorption onto aluminium hydroxide.

Antibodies

A panel of nine murine monoclonal antibodies, which were raised against serum-derived HBsAg and shown to bind to the common "a" determinants of HBsAg [Waters et al., 1991], were used in this study. The ascitic fluid of each monoclonal antibody, used to coat the solid phases, when tested using an antigen-specific enzyme-linked immunosorbent assay (ELISA), had a specific antibody titre of greater than 1/10,000. The immunoglobulin G1 (IgG1) monoclonal antibodies which were to be radiolabelled were purified from ascitic fluid on a Protein A-Sepharose column (Pharmacia, Uppsala, Sweden), as described previously [Goodall et al., 1982].

Epitope Mapping

Monoclonal antibodies, in the form of ascitic fluid, were coated onto polystyrene beads (Northumbria Biologicals, Cramlington, UK) at a dilution of 1/100 in bicarbonate buffer, pH 9.6, for 1 hr at room temperature and for 16 hr at 4°C [Goodall et al., 1982].

Protein A-Sepharose-purified monoclonal antibody RFHBs 18 was radiolabelled with sodium iodide I-125 (Amersham, Aylesbury, UK) using the chloramine T method as described previously [Goodall et al., 1982].

Coated beads were incubated with the vaccine samples diluted in 50% newborn calf serum (NBCS)/phosphate-buffered saline (PBS) for 16 hr at room temperature. Beads were washed and the antigen bound was detected by incubating with 100,000 counts/minute (cpm) radiolabelled RFHBs 18 for 2.5 hr at 37°C.

Each assay was undertaken using a range of concentrations of Hep B-3, from 100 ng/ml to 0.1 ng/ml. A standard curve was prepared, using each of the solid phases in every assay, with a plasma pool calibrated against the WHO International Standard for HBsAg (NIBSC 80/549). Five negative controls of the diluent plus the labelled monoclonal antibody alone were included in each assay.

Specificity of Antibodies Induced in Vaccinees

Serum was collected at months 0, 1, 2, and 3 from a group of naive vaccinees, six female and seven male, who completed the full course of three immunisations at months 0, 1, and 2. Each immunisation consisted of 20μg Hep B-3 formulated with aluminium hydroxide. The epitopes recognised by the antibodies present in their sera were investigated.

Polystyrene beads were coated, as above, with 1 μg/ml of HBsAg purified from the plasma of a patient chronically infected with HBV. Protein A-Sepharose-purified monoclonal antibodies RFHBs 1, 7, and 18 (10 μg) were radiolabelled using the chloramine T method as described previously [Goodall et al., 1982].

A washed, coated bead was coincubated with 100 μl of each serum sample from the vaccinees and 100 μl of a radiolabelled monoclonal antibody, either RFHBs 1, RFHBs 7, or RFHBs 18, diluted in 50% NBCS/PBS to contain 100,000 cpm. The reagent was incubated for 2 hr at room temperature, and the beads were washed and counted. Preimmunisation samples were used as negative controls. Inhibition by dilutions of purified, unlabelled "self" monoclonal antibodies were used as positive controls.

The total anti-HB titres in the vaccinee serum were measured using a commercial assay (IMx; Abbott Laboratories, North Chicago, IL). Antibody titres were confirmed using the AUSAB anti-HBs assay (Abbott Laboratories).

Statistical Analysis

The probability of a correlation between the percentage inhibition of each of the monoclonal antibodies and the total anti-HB titre in the serum was calculated using the Spearman rank test.

RESULTS

Epitope Mapping of Vaccine Preparations Using a Panel of Monoclonal Antibodies

Five batches of vaccine were tested simultaneously using the panel of monoclonal antibodies on the solid

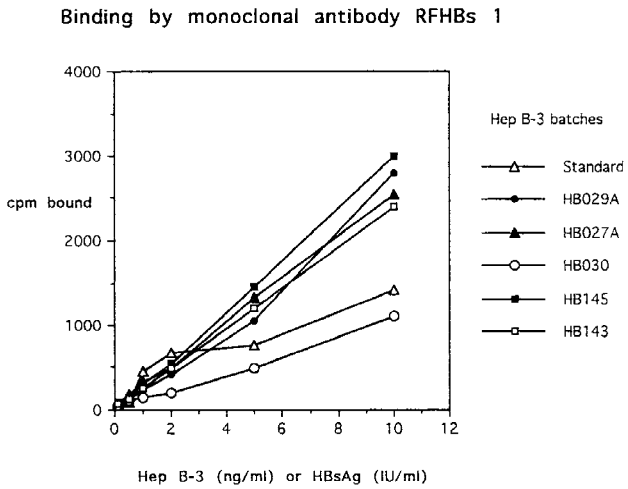

Fig. 1. Epitope mapping of the batches of Hep B-3; binding curves of the batches tested using RFHBs 1-coated solid phase.

phase to assess the HBsAg epitopes present on the antigen over a concentration range of 0.1–100 ng/ml; in the same assay, the standard serum HBsAg preparation was tested over a range of 0.1–20 IU/ml. Using RFHBs 18 on the solid phase and as the radiolabeled without inhibiting binding to the antigen is possible since recombinant HBsAg, as serum-derived HBsAg, self-assembles into particles containing multiple polypeptides.

The cpm bound over an antigen concentration of 0–12 ng/ml, when using the solid phase coated with RFHBs 1, of each of the preparations and of the serum standard is illustrated in Figure 1. This figure illustrates the linear part of the binding curve.

The results also were calculated as a ratio of the cpm in the test wells to the cpm in the negative controls. Table I illustrates the results expressed as positive to negative ratios at an antigen or protein concentration of 5 IU/ml or 5 ng/ml. Similar comparisons could have been made at other concentrations of the antigen, but this was the most sensitive point to evaluate any differences as it is on the linear part of the standard curve for each of the solid phases used.

Comparison of the five batches of vaccine, HB 027, 029, 030, 145, and 143, showed very little batch to batch variation. The monoclonal antibodies on the solid phase detected the vaccine preparations with the same sensitivity as the standard serum-derived preparation. One of the preparations, HB 030, was bound less well by the monoclonal antibody solid phases than the other batches. Visual examination of this preparation showed that some flocculation had occurred on storage, probably resulting from the reduction of antigenic sites available to the antibodies.

Specificity of Antibodies Induced in Vaccines

The percentage inhibition of the binding of each of three monoclonal antibodies to serum-derived HBsAg-coated solid phase by vaccinee sera taken at month 3 was calculated in comparison to the inhibition of binding of the antibodies by preimmune sera (Table II). As a control, the inhibition of each label by unlabelled Protein A-Sepharose-purified "self" antibody in the same assay also was calculated (Table III).

The antibody which recognised the same epitope as RFHBs 1, the monoclonal antibody known to be protective in chimpanzees [Iwarson et al., 1985], was raised in all but one of the vaccinees. In this group of vaccinees, the percentage inhibition of this antibody significantly correlated with the anti-HBs titre as analysed using the Spearman rank correlation ($P = 0.0041$). The induction of anti-HBs was confirmed using the AUSAB assay. Serum from vaccinee 116 did not inhibit the binding of monoclonal antibodies despite having a similar titre of anti-HBs to vaccinee 115 in the IMx assay and a titre of greater than 150 IU/l using the AUSAB assay. These results suggest that anti-HBs was raised in vaccinee 116 against epitopes other than those recognised by the monoclonal antibodies used in this study.

The inhibition of RFHBs 7 correlated with the total anti-HBs titre ($P = 0.0174$), and the inhibition of RFHBs 18 correlated with the total anti-HBs titre ($P = 0.0065$). The correlations are illustrated in Figure 2.

The percentage inhibition of RFHBs 1 by the sera was greater than that of RFHBs 7 or 18. This is similar to results obtained previously by testing the sera of vaccinees immunised with plasma-derived vaccine and a vaccine produced in yeast containing only the S gene [Waters et al., 1987].

DISCUSSION

The region between amino acids 110 and 160 of the small HBsAg is thought to be important for inducing an antibody response which is protective. It is in this region that the common "a" determinant is thought to lie. Several strands of evidence suggest that the conformation of the "a" determinant is important for its immunogenicity.

Early experiments with 22 nm HBsAg particles from serum demonstrated that destruction of the disulphide bonds of HBsAg partially destroyed its antigenicity [Imai et al., 1974]. In addition, intact disulphide bonds were required to maintain antigenicity in tryptic digests [Burrell et al., 1976]. The region between amino acids 110 and 160 is rich in cysteine molecules, suggesting that it is important to the conformation of the protein and, therefore, to its antigenic structure. Computer modelling of HBsAg suggested that this region, present on the surface of the viral and 22 nm particles, is more hydrophilic than other parts of the polypeptide [Howard et al., 1988].

The recombinant vaccine Hep B-3 also contains the pre-S2 and the pre-S1 regions, which have important helper T-cell epitopes [Jin et al., 1988; Ferrari et al., 1989]. In congenic mice, these regions have been shown to augment the anti-S response and to circumvent nonresponsiveness to the S region [Milich, 1988]. In a human trial in a group of nonresponders to the S-region

TABLE I. Epitope Mapping of Five Batches of Hep B-3 Using a Panel of Monoclonal Antibodies on the Solid Phase

Monoclonal antibody	Positive/negative ratio at 5 ng/ml of antigen					
	Standard	HB027	HB029	HB030	HB143	HB145
RFHBs 1	11	21.7	17.3	8.2	19.8	23.9
RFHBs 2	2.4	1.4	2.1	1.3	2.8	2.4
RFHBs 4	13	11.6	19.4	9.0	15.5	18.9
RFHBs 7	0.9	10.3	10.4	5.0	15.4	13.4
RFHBs 13	13.3	16.4	18.7	8.5	15.2	16.6
RFHBs 14	62.5	90	75.8	42.2	85	87.4
RFHBs 16	40.5	59.6	49.3	14.5	42.7	44.7
RFHBs 18	11.9	11.8	10.8	7	14.2	13
RFHBs 20	48	61.9	57.6	26.6	63.5	24.8

TABLE II. Inhibition of Radiolabelled Monoclonal Antibodies by Vaccinee Sera

Vaccinee	Total-anti-HBs (IU/L)	% Inhibition		
		RFHBs 1	RFHBs 7	RFHBs 18
102	4,300	97	0	14
105	4,520	97	37	53
110	1,840	95	7	16
112	148	63	3	2
115	36	26	2	11
116	47	0	0	0
119	2,680	82	11	33
204	1,880	92	10	6
206	2,240	92	6	42
207	1,170	93	9	30
213	4,660	95	21	52
220	1,550	79	0	28
221	9,150	97	53	68

Mean binding of monoclonal antibodies in the presence of preimmunisation sera. 0% inhibition RFHBs 1, 10,926 cpm; 0% inhibition RFHBs 7, 12,221 cpm; 0% inhibition RFHBs 18, 29,972 cpm.

TABLE III. Inhibition of Labelled Monoclonal Antibodies by Unlabelled "Self" Antibodies

Antibody concentration (μg/ml)	% Inhibition by unlabelled "self" antibody		
	RFHBs 1	RFHBs 7	RFHBs 18
10	95	92	90
5	86	87	82
2	68	77	76
1	39	59	56
0.1	22	0	0

vaccines, 69% became anti-HBs-positive with a titre of greater than 10 IU/l [Zuckerman et al., 1997].

It is important to test any new vaccine to ensure that the important "a" determinants are intact and that the immunogenicity of the S region remains unaffected.

The antigenicity of HBsAg has been mimicked in part using cyclic peptide analogues of amino acids 124–137 and 139–147. Although antibodies in both vaccinee and convalescent sera bound to linear peptide analogues, they did so with a much reduced affinity [Brown et al., 1984]. The monoclonal antibodies used in this study have been characterised previously [Waters et al., 1991]. They all bind to the common "a" determinants and have been subdivided by their ability to bind to the cyclical peptide analogues of amino acids 124–137 (p124–137) and 139–147 (p139–147) of HBsAg. RFHBs 1, 2, and 4 bind to the cyclical peptide p124–137. RFHBs 2 and 4 also partly compete with each other, suggesting that the two epitopes recognised by these antibodies are closely related. RFHBs 1 has been shown to neutralise the infectivity of a standard virus inoculum in an animal model [Waters et al., 1987]. The epitopes recognised by these antibodies are well represented in Hep B-3 and compare favourably with the serum-derived HBsAg control.

RFHBs 7, 14, and 16 bind to p139–147 of the 25,000 dalton polypeptide. Three other antibodies, RFHBs 13, 18, and 20, do not bind to either peptide analogue, although RFHBs 7 and 18 partially compete with each other and with RFHBs 4. All of these epitopes are well represented in Hep B-3 and compare favourably with the plasma-derived preparation.

Using one monoclonal antibody, RFHBs 1, which binds to p124–137, a second antibody, RFHBs 7, which binds to p139–147, and a third antibody, RFHBs 18, which binds to neither peptide but does recognise a common "a" determinant, antibody specificity induced in vaccine recipients was tested. Vaccination induced appreciable amounts of antibody recognising the same epitope as the virus-neutralising antibody RFHBs 1, and this was in proportion to the total anti-HBs titre induced. This is in accord with previous results obtained using both a plasma-derived vaccine and a recombinant vaccine prepared in yeast [Waters et al., 1987]. In addition to inducing high titres of the known neutralising antibody, however, antibodies which recognised the same epitopes as both RFHBs 7 and 18 were induced in the vaccinees in proportion to the total anti-HBs titre. The immunisation schedule used in this trial in naive subjects was shorter than that used in the plasma-derived and recombinant vaccine trials in which the antibody specificity was examined previously. A more recent trial in nonresponders [Zuckerman et al., 1997] has demonstrated efficacy and immunogenicity after two doses of vaccine. All of these results suggest that the inclusion of pre-S1 and pre-S2 antigenic sequences have improved the immunogenicity of the vaccine.

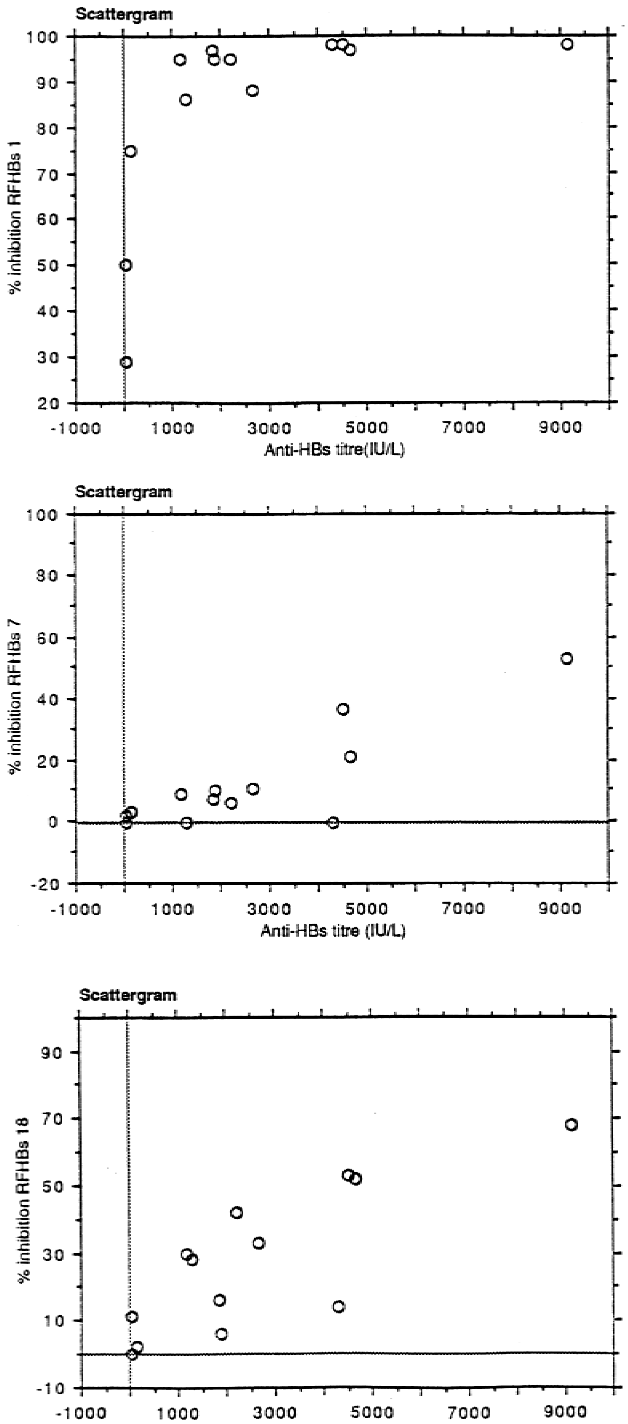

Fig. 2. Scattergrams depicting the correlation between anti-HBs, as measured by the IMx assay, in vaccinee sera and the percentage inhibition of the monoclonal antibodies RFHBs 1, 7, and 18 by these sera.

REFERENCES

Brown SE, Howard CR, Zuckerman AJ, Steward MW (1984): Affinity of antibody responses in man to hepatitis B vaccine determined with synthetic peptides. Lancet ii:184–187.

Burrell CJ, Leadbetter M, Mackay P, Marmion BP (1976): Intact disulphide bonds are required for full expression of serological activity when the "a" determinant binding site is cleaved from the rest of the HBsAg protein. Journal of General Virology 33:41–50.

Ferrari C, Penna A, Bertoletti A, Cavalli A, Valli A, Schianchi C, Fiaccadori F (1989): The preS1 antigen of hepatitis B virus is highly immunogenic at the T cell level in man. Journal of Clinical Investigation 84:1314–1319.

Gerety RJ (1988): Recombinant hepatitis B vaccines. In Zuckerman AJ (ed): "Viral Hepatitis and Liver Disease." New York: Alan R Liss, pp 1017–1024.

Goodall AH, Meek FL, Waters JA, Miescher GC, Janossy G, Thomas HC (1982): A rapid one-step radiometric assay for hepatitis B surface antigen utilising monoclonal antibodies. Journal of Immunological Methods 52:167–174.

Howard CR, Brown SE, Hogben DN, Zuckerman AJ, Murray-Lyon IM, Steward MW (1984): Analysis of antibody responses to hepatitis B surface antigen. In Vyas GN, Dienstag JL, Hoofnagle JH (eds): "Viral Hepatitis and Liver Disease." New York: Grune and Stratton, pp 561–572.

Howard CR, Stirk HJ, Brown SE, Steward MW (1988): Towards the development of synthetic hepatitis B vaccines. In Zuckerman AJ (ed): "Viral Hepatitis and Liver Disease." New York: Alan R Liss, pp 1094–1101.

Imai M, Gotoh A, Nishioka K, Kurashima S, Miyakawa Y, Mayumi M (1974): Antigenicity of reduced and alkylated Australia antigen. Journal of Immunology 112:416–419.

Iwarson S, Tabor E, Thomas HC, Goodall A, Waters J, Snoy P, Shih JW-K, Gerety RJ (1985): Neutralization of hepatitis B virus infectivity by a murine monoclonal antibody: an experimental study in the chimpanzee. Journal of Medical Virology 16:89–96.

Jilg W, Lorbeer B, Schmidt H, Wilske B, Zoulek G, Deinhardt F (1984): Clinical evaluation of a recombinant hepatitis B vaccine. Lancet i:1174–1175.

Jin Y, Shih WK, Berkower I (1988): Human T cell response to the surface antigen of hepatitis B virus (HBsAg). Endosomal and non-endosomal processing pathways are accessible to both endogenous and exogenous antigen. Journal of Experimental Medicine 168: 293–306.

Koziol DE, Alter HJ, Kirchner JP, Holland PV (1976): The development of HBsAg positive hepatitis despite the previous existence of antibody to HBsAg. Journal of Immunology 117:2260–2262.

Le Bouvier GH, Capper RH, Williams AE, Pelletier M, Katz AJ (1976): Concurrently circulating hepatitis B surface antigen and heterotypic anti-HBs antibody. Journal of Immunology 117:2262–2264.

McAleer WJ, Buynak EB, Maigetter RZ, Wampler DE, Miller WJ, Hilleman MR (1984): Human hepatitis B vaccine from recombinant yeast. Nature 307:178–180.

Milich DR (1988): T- and B-cell recognition of hepatitis B viral antigens. Immunology Today 9:380–386.

Mishiro S, Imai M, Takahashi K, Machido A, Gotando T, Miyakawa Y, Mayumi M (1980): A 49,000 dalton polypeptide bearing all antigenic determinants and full immunogenicity of 22nm hepatitis B surface antigen particles. Journal of Immunology 124:1589–1593.

Pavlakis GN, Hamer DH (1983): Regulation of a metallothienin-growth hormone hybrid gene in bovine papilloma virus. Proceedings of the National Academy of Sciences 80:387–401.

Samonta H, Youn BW (1989): Expression of hepatitis B virus surface antigen containing the pre-S region in mammalian cell culture system. Vaccine 7:69–76.

Szmuness W, Stevens CE, Harley EJ, Zang EA, Oleszko WR, William DC, Sadovsky R, Morrison JM, Kellner A (1980): Hepatitis B vaccine. Demonstration of efficacy in a controlled clinical trial in a high-risk population in the United States. New England Journal of Medicine 303:833–841.

Valenzuela P, Medina A, Rutter WJ, Hammerer G, Hall BD (1982): Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. Nature 298:347–350.

Waters J, Pignatelli M, Galpin S, Ishihara K, Thomas HC (1987a): Virus-neutralising antibodies to hepatitis B virus: The nature of an immunogenic epitope on the S gene peptide. Journal of General Virology 67:2467–2473.

Waters JA, O'Rourke SM, Richardson SC, Papaevangelou G, Thomas HC (1987b): Qualitative analysis of the humoral immune response

to the "a" determinant of HBs antigen after inoculation with plasma-derived or recombinant vaccine. Journal of Medical Virology 21:155–160.

Waters JA, Brown SE, Steward MW, Howard CR, Thomas HC (1991): Analysis of the antigenic epitopes of hepatitis B surface antigen involved in the induction of a protective antibody response. Virus Research 22:1–12.

Yoneyama T, Akatsuka T, Miyamura T (1988): Stable expression of the hepatitis B virus surface antigen containing pre-S2 protein in mouse cells using a bovine papillomavirus vector. Journal of General Virology 69:1931–1939.

Zuckerman JN, Sabin C, Craig FM, Williams A, Zuckerman AJ (1997): Immune response to a new hepatitis B vaccine in healthcare workers who had not responded to standard vaccine: Randomised double blind dose-response study. British Medical Journal 314:329–333.